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Distinctive traits of normal and tumor-derived human mammary epithelial cells expressed in a medium that supports long-term growth of both cell types

(DFCI-1 medium/primary breast tumors/tumor antigens/rhodamine 123)

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ABSTRACT **A medium is described that supports long-term growth in culture of human primary mammary tumor cells, of normal epithelial cells from mammoplasty, and of mammary tumor cell lines. Tumor cells are shown to be distinguishable from normal mammary epithelial cells by morphology, by growth requirements, and by two markers: preferential expression of the HMFG-2 epitope on tumor cells and preferential retention in tumor cell mitochondria of the lipophilic fluorescent dye rhodamine 123. Differential fluorescence of HMFG-2 fluorescein-conjugated antibodies can be used as a basis for separation of normal and tumor cells in a fluorescence-activated cell sorter, as can differential retention of rhodamine 123.**

Breast cancer is one of the most frequent lethal malignancies in women, yet it remains among the least studied and most poorly understood of all human cancers. A major reason has been the difficulty in growing either normal or tumor-derived human mammary epithelial cells in culture (1–3). Even the establishment of tumor cell lines has been an infrequent accomplishment (4–6). A related problem has been the difficulty in distinguishing in culture between primary tumor cells and their normal epithelial progenitors after tissue specimens are disaggregated. No generally accepted cell culture tumor markers have been available.

We have begun an investigation of human breast oncogenesis at the cellular and molecular levels with two principal aims: (*i*) to identify early changes in neoplastic cells that would further our understanding of the origin and diverse properties of primary breast tumors, a study aimed in part at finding new prognostic markers for stage I and stage II disease; and (*ii*) to isolate genes whose products have a growth-inhibitory or tumor-suppressor function in this system.

An essential requirement for these studies is the ability to grow and identify primary breast tumor cells in long-term culture so that early tumorigenic changes in gene expression can be investigated. Conventionally, mammary tumor cell lines have been isolated from metastases or pleural effusions and grown in medium containing standard salts (e.g., Eagle's minimum essential medium, MEM) with 10% fetal bovine serum (4–6), whereas normal mammary epithelial cells have been grown in MCDB 170, a serum-free medium containing bovine pituitary extract (7). As we report here, primary tumor cells as well as established tumor cell lines do not grow in MCDB 170, and, as is well known, epithelial cells in surgical specimens are irrevocably overgrown by fibroblasts in media with high serum.

Although growth of primary breast tumor cells has been a goal of many investigators, only one serum-free medium, CDM3, was recently described that supports their growth, albeit short-term (8). No medium has been available for the long-term culture of primary tumor cells, and as a consequence little is known about them.

This paper reports three results. (*i*) DFCI-1 medium is described; this low-serum medium was used for the successful isolation and long-term culture of normal mammary epithelial cells from mammoplasties and of primary and metastatic mammary tumor cells.

(*ii*) Two markers are shown to distinguish normal cells from tumor cells when both are grown in DFCI-1 medium: expression of the HMFG-2 antigenic determinant (9–11) on tumor cells but not on normal cells, and preferential retention of the dye rhodamine 123 (R-123) in tumor cells (12, 13). Monoclonal antibody HMFG-2 recognizes an epitope on human milk fat globules (10) that is expressed more strongly on sections of breast tumors than on sections of normal breast tissue (11). R-123, a fluorescent lipophilic cationic dye that readily enters the mitochondria of living cells (12), is retained longer within some human tumor cells, including several mammary tumor cell lines, than in normal epithelial cells from monkey kidney used as controls (13).

(*iii*) Evidence is presented that normal and tumor cells growing side by side in a primary tumor explant can be distinguished, separated, and grown independently in DFCI-1 medium. Pure cultures of each have been distinguished by morphology, by HMFG-2 binding, by R-123 retention, and by growth in different media.

MATERIALS AND METHODS

Tissue Collection and Culture. Normal human mammary tissue specimens from reduction mammoplasties and biopsy samples were received in sterile tubes containing α-MEM with 10% fetal bovine serum and antibiotics. Tissue was cut with a tissue chopper (Mickle Laboratory Engineering, Gomshall, Surrey, U.K.) into ≈1-mm pieces and then either frozen in medium containing 10% dimethyl sulfoxide and 20% fetal bovine serum or cultured in flasks in DFCI-1 medium at 37°C in a humidified atmosphere of 6.5% CO_2. Trypsinization was carried out as described (7, 14).

Cell Strains and Cell Lines. Strains 184 and 172 were derived from reduction mammoplasties (7, 15). The ZR-75-1-2-T (here called ZR-T) cell line was derived in our laboratory from a nude mouse tumor produced by a precloned derivative, ZR-75-1-2, of ZR-75-1 cells obtained from the American Type Culture Collection. The MCF7T cell line was from a nude mouse tumor derived from MCF7 cells (Michigan Cancer Foundation). MDA-MB-231, BT-20, T-47D, SK-BR-3, HBL-100, BT-549, and MDA-MB-436 cells

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Abbreviations: R-123, rhodamine 123; CEA, carcinoembryonic antigen.

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were obtained from the American Type Culture Collection. 21N and 21PT were mastectomy samples from a patient with infiltrating ductal and intraductal carcinoma. Tissues were processed as above and grown in DFCI-1 medium. Fibroblasts were removed by trypsinization with cold (4°C) trypsin.

Media. *DFCI-1.* This medium consists of α-MEM/Ham's nutrient mixture F-12 (1:1, vol/vol) supplemented with 12.5 ng of epidermal growth factor per ml, 10 nM triiodothyronine, 10 mM Hepes, 50 μM freshly made ascorbic acid, 2 nM estradiol, 1 μg of insulin per ml, 2.8 μM hydrocortisone, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, 10 μg of transferrin per ml, 2 mM L-glutamine, 100 units of penicillin per ml, and 100 μg of streptomycin sulfate per ml (all from Sigma); 15 nM sodium selenite (Amend Drugs and Chemical, New York); 1 ng of cholera toxin per ml (Schwarz/Mann); 1% fetal bovine serum (J. R. Scientific, Woodland, CA or Hyclone); and 35 μg of bovine pituitary extract per ml (Hammond, Cell/Tech, Alameda, CA). The pH was 7.4 at 6.5% CO_2.

MCDB 170. The liquid formulation of MCDB 170 was obtained from the Cell Culture Facility, University of California at San Francisco, and supplemented as described (7, 14).

α medium. This consists of α-MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM Hepes, and 100 units of penicillin and 100 μg of streptomycin sulfate per ml.

Determination of Cell Growth. To measure growth in DFCI-1 medium, cells were washed once with solution A (a balanced salts solution) (7) and trypsinized (0.025% trypsin plus 0.01% EDTA; Sigma). Trypsin digestion was stopped with 0.0375% soybean trypsin inhibitor (Sigma) in solution A, and cells were washed and counted in a Coulter Counter. Approximately 5×10^4 cells were plated per 35-mm dish (Falcon) and grown at 37°C in a humidified atmosphere with 6.5% CO_2. The population doubling times were estimated in triplicate from the linear portion of the curve.

Immunofluorescence Staining. Murine monoclonal antibodies to DF3 (16), keratin (17), and HMFG (11) antigens were kindly provided by Donald Kufe (this institute), Lan Bo Chen (this institute), and Joyce Taylor-Papadimitriou (Imperial Cancer Research Fund, London), respectively. Rabbit antibodies against carcinoembryonic antigen (CEA) were from Chemicon. Exponentially growing cells were trypsinized, resuspended in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) and distributed at 10^5 cells per well in 96-well round-bottom plates (Linbro). For keratin staining, cells were fixed with methanol for 2 min at −20°C and washed once with water and three times with PBS. For surface antigens DF3, CEA, and HMFG, cells were immunostained without fixation. Cells were incubated with 50 μl of antibody diluted in PBS (anti-DF3 at 1:100, anti-CEA at 1:50, J1B3 at 1:40, or HMFG-2 undiluted) for 45 min on ice. Cells were then washed three times with PBS containing 1% BSA and were incubated with fluorescein isothiocyanate (FITC)-labeled $F(ab')_2$ goat anti-mouse IgG (for DF3, J1B3, and HMFG-2) or FITC-labeled $F(ab')_2$ goat anti-rabbit IgG (for CEA) (Tago) for 45 min on ice. The negative control was either rabbit IgG (for CEA) (Chemicon) or P3 ascites secreted by P3X63.Ag8 (IgG1; diluted 1:100) (18). Cells were then washed three times with PBS plus 1% BSA and resuspended in 500 μl of PBS plus 1% BSA. The fluorescence was measured on an Epics IV cytofluorograph using an excitation wavelength of 488 nm and recording the emission at 515 nm. Using this method one can measure fluorescence intensity over a range spanning 3 logarithmic units (19).

Measurement of DNA Content by Cytofluorography. DNA content was measured as described (20). In brief, confluent cultures of cells ($\geq 10^6$ per 25-cm^2 flask) were trypsinized as above, washed once with PBS, and fixed in 50% methanol. The cells were pelleted by centrifugation and resuspended in 1 ml of 15 mM $MgCl_2$ containing chromomycin A3 at 20 μg/ml; they were kept in the dark until fluorescence was measured at 457 nm with an Epics IV cytofluorograph.

Retention of R-123. A freshly made stock solution (1 mg/ml) of R-123 (Eastman Kodak) in dimethyl sulfoxide was added to exponentially growing cells to give a final concentration of 10 μg/ml in culture media. After incubation for 1 hr, cells were washed three times (10 min each) with respective media and then incubated further at 37°C for 48 hr. Cells then were washed once with PBS, released with trypsin, and resuspended in 500 μl of PBS. R-123 fluorescence was measured with an Epics IV cytofluorograph using an excitation wavelength of 488 nm and recording at 515 nm.

RESULTS

Establishment of Normal Mammary Epithelial Cell Strains in DFCI-1 Medium. Epithelial cell colonies were visible within 3–5 days after mechanically chopped material from mammoplasty specimens was seeded into flasks containing DFCI-1. In the majority of samples (70N, 76N, 81N, 83N, and 86N), remaining fibroblasts were largely removed at passage 2 by incubating the cultures with cold trypsin for 2–3 min. Cultures were trypsinized when about 70% confluent and split 1:3. Fibroblast cultures were also established in α medium from each mammoplasty specimen for comparative studies.

Epithelial cell populations from mammoplasties appear as homogeneously small cells in the first passage. In the next few passages, both large and small cells are present. At about passage 5, selection occurs: the large cells become vacuolated and stop dividing, whereas small cells overgrow the culture. In subsequent passages, the population consists of uniform small cells that resemble the small cells initially seen (Fig. 1 *e* and *f*). This selection process is similar to that described by Hammond *et al.* (7).

Mammoplasty-derived cells grown in DFCI-1 medium were harvested when confluent at passage 3 or 4 to determine DNA content. The DNA content of each strain was found to be 2*N* (data not shown) by cytofluorography using chromomycin A3 (20). One strain (70N) was karyotyped by Giemsa banding and found to be diploid with 46XX chromosomes (C. Morton, unpublished observation).

Isolation and Growth of Primary Breast Tumor Cells in DFCI-1. Two biopsy samples, 21N (from surrounding normal tissue) and 21PT (from primary tumor), from a mastectomy were recovered from surgery, minced as described, and grown in DFCI-1 medium. The 21N tissue explant exhibited similar growth characteristics (Fig. 1 *a* and *b*) as described above for mammoplasty-derived normal epithelial cells. The 21PT sample grew more slowly initially. Fibroblasts were removed with cold (4°C) trypsin or by EDTA. By the third passage (about 5 weeks) there were mainly epithelial cells of two distinct morphologies (Fig. 1 *a–d*). Those considered normal resembled the mammoplasty specimens (Fig. 1 *e* and *f*) and the 21N sample (Fig. 1 *a* and *b*) in their slightly elongated spindle shape and close packing in clusters of growing cells. The putative tumor cells, on the other hand, were polygonal, loosely arranged, and heterogeneous in cell size (Fig. 1 *c* and *d*).

Cell Growth in DFCI-1 Medium. Fig. 2*a* shows the growth kinetics of six normal epithelial cell strains in DFCI-1 medium. All the cells were used at passage 3 or 4 except for cells of strain 184, which were at passage 9. These strains show exponential growth in DFCI-1 with population doubling

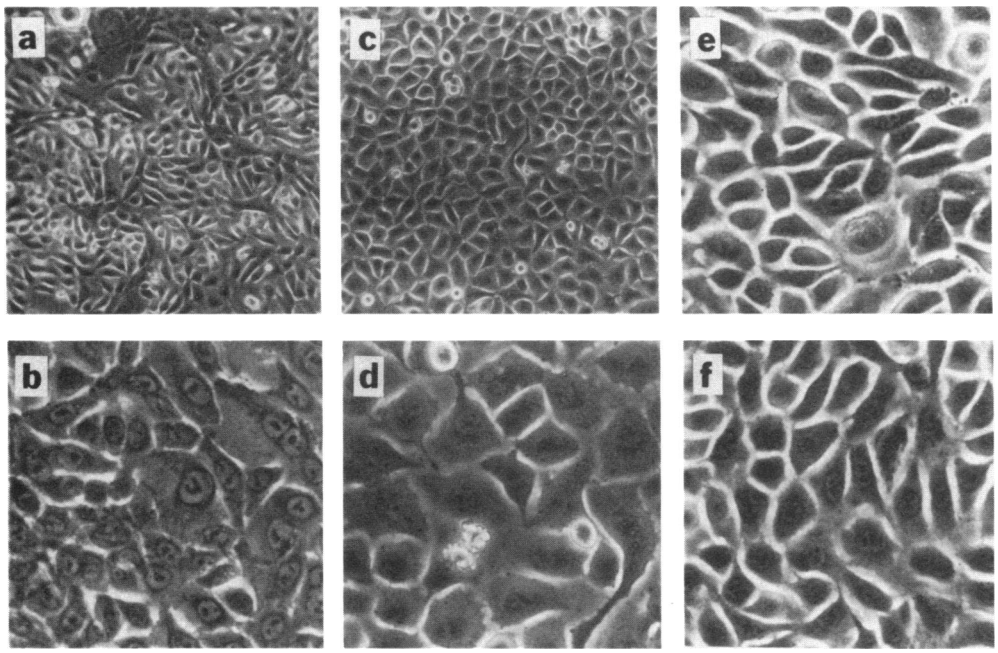

FIG. 1. Morphology of normal and tumor cells established in DFCI-1. Light micrographs of normal and primary tumor-derived epithelial cells as shown by phase-contrast optics. (*a* and *b*) Normal epithelial cells from 21N at low and high magnifications (×70 and ×230). (*c* and *d*) Tumor epithelial cells from 21PT (×70 and ×230). (*e* and *f*) Normal epithelial cells from mammoplasty specimens 70N and 76N (×230).

times of 19–34 hr. The two mammoplasty strains (76N and 70N) that were established are now in passages 10–20, with consistent doubling times of 22–24 hr. Cell strains 184 and 172 have been carried to passage 21 in DFCI-1 medium before they senesced. The established mammary epithelial tumor cell lines MCF7T, ZR-T, and MDA-MB-231 grow with population doubling times of 28–40 hr in DFCI-1 (Fig. 2*b*) and have been maintained in this medium for ≥10 passages. Other mammary carcinoma cell lines including BT-20, SKBR-3, MDA-MB-436, and T-47D also have been grown in this medium.

Growth studies of 184, 172, 21N, 21PT, MCF7, ZR-75-1, and MDA-MB-231 cells in MCDB 170, DFCI-1, and α media showed that normal cells grow well only in MCDB 170 and DFCI-1, whereas 21PT and tumor cell lines grow in DFCI-1 and α media but not in MCDB 170 (Table 1).

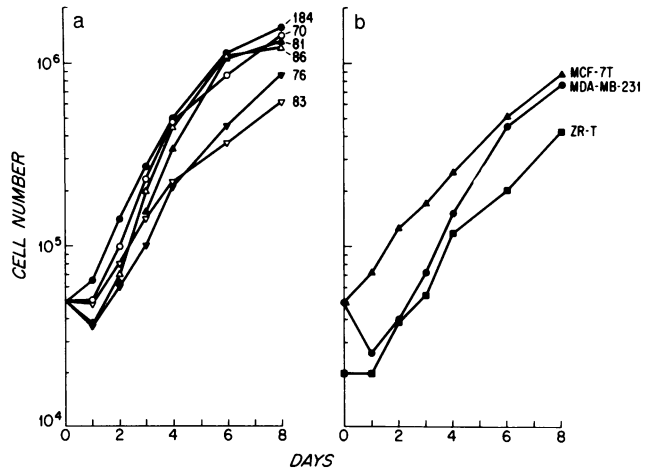

FIG. 2. Growth of mammary epithelial cells in DFCI-1 medium. (*a*) Normal cells 184, 70N, 81N, 86N, 76N, and 83N. (*b*) Established cell lines MCF7T, MDA-MB-231, and ZR-T. Approximately 5×10^4 or 2×10^4 (ZR-T) cells were seeded per 35-mm culture dish. At the indicated times, cells were harvested and counted in a Coulter Counter. Each data point represents mean of at least three dishes. Normal cells were seeded at passage 3 or 4; strain 184 cells were at passage 9 (three passages in DFCI-1). Tumor cell lines MDA-MB-231, MCF7T, and ZR-T were at passages 3, 5, and 10, respectively.

Antigen Expression on Normal and Tumor Cells. Breast carcinoma-specific antigens have long been sought for purposes of diagnosis and therapy. Despite intensive efforts in many laboratories, few diagnostically successful antibody preparations have been reported (21). Of particular interest to us are antibodies that could be used with growing surgical explants to distinguish between normal and tumor cells.

Initially we compared antigens on normal cells grown in MCDB 170 with tumor cells grown in α medium. Significant differences were observed in binding of antibodies against DF3, CEA, and HMFG-2. When DFCI-1 medium became available, the studies were repeated to assess possible effects of the growth medium. An example of the results is shown in Fig. 3. We compared the normal 184 cells grown in MCDB 170, in MCDB 170 plus 1% fetal bovine serum, and in DFCI-1 (Fig. 3 *a–c*, respectively), as well as MCF7 (Fig. 3*d*) and ZR-T (Fig. 3*e*) cells grown in DFCI-1 medium.

The medium strongly influenced antigen expression of DF3 and CEA in normal cells. This is shown as a rightward shift in fluorescence in Fig. 3 *b* and *c* compared to *a*, as well as in comparison to controls without specific antibodies, prerun in each experiment. In contrast, the control and treated 184 cells bound the same baseline amounts of HMFG-2 regardless of the medium (*a–c*). The results with the tumor cells (*d* and *e*) grown in DFCI-1 medium show that HMFG-2 binding is extensive (53% and 63%) compared to the normal cells grown in the same medium (1%). Thus the medium does exert an influence on DF3 and CEA antigen expression. Of the three candidate antibody preparations tested, only HMFG-2 was differentially bound by normal and tumor cells grown under the same conditions. Normal cell strains grown from mammoplasty specimens in DFCI-1 behaved similarly to the

Table 1. Growth of normal and tumor cells in various media

Cells	DFCI-1	MCDB 170	α medium
184	+	+	−
172	+	+	−
21N	+	+	−
21PT	+	−	+
MCF7	+	−	+
ZR-75-1	+	−	+
MDA-MB-231	+	−	+

+, Growth; −, no growth.

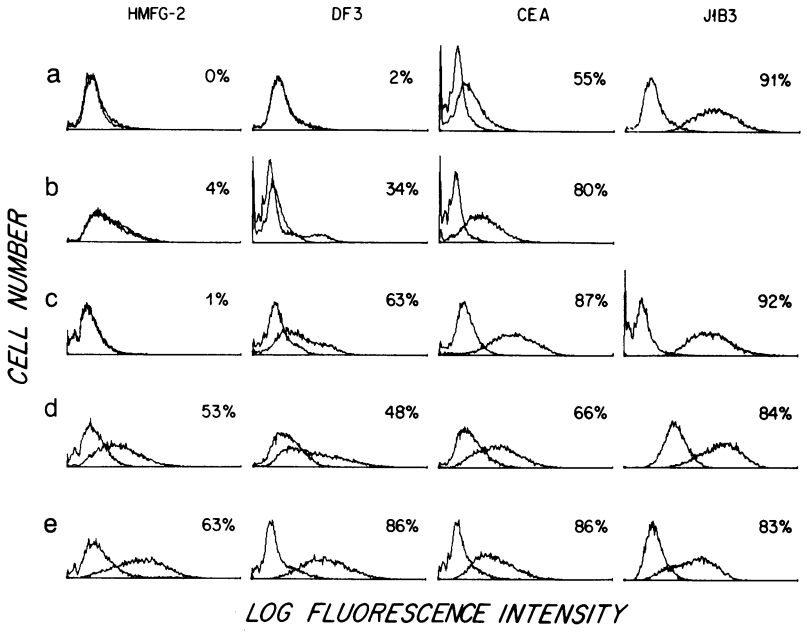

FIG. 3. Effect of medium on antigen expression in normal 184 cells and tumor cell lines MCF7 and ZR-T. Cells were treated with antibodies against HMFG-2 antigenic determinant, DF3, CEA, and keratin (J1B3) and with control antibodies as described in *Materials and Methods*. (*a–c*) Strain 184 cells grown in serum-free MCDB 170, MCDB 170 with 1% fetal bovine serum, or DFCI-1, respectively. (*d* and *e*) MCF7 and ZR-T cells, respectively, grown in DFCI-1. Fluorescence of cells stained with various antibodies is shown as a rightward shift in comparison to fluorescence obtained with control antibodies. The percent fluorescence-shifted cells is given in each histogram.

184 cells for the expression of keratin (J1B3), DF3, CEA, and HMFG-2 antigenic determinants (data not shown).

We have examined HMFG-2 binding on a wide range of normal epithelial cells grown in this laboratory: mammoplasty-derived cells (strains 30N, 56N, 70N, 76N, 81N, 83N, 86N, 184, and 172); cells from normal tissue of breast cancer patients (strains 21N, 27N, 28N, and 58N); and tumor cell lines (ZR-75-1, MCF7, BT-20, T-47D, SK-BR-3, HBL-100, and BT-549). All tumor cell lines were found to bind HMFG-2, whereas none of the normal epithelial cell strains bound it (data not shown).

To further confirm that 21PT-derived epithelial cells were mammary tumor epithelial cells, we compared HMFG-2 binding on 21PT, 21N, and 76N. As shown in Fig. 4, a high percentage (87%) of 21PT cells express the HMFG-2 epitope as compared to 21N (7%) and 76N (4%).

Retention of R-123. Normal 184 cells and other mammoplasty-derived strains retain less dye when grown in MCDB 170 (peak 1, Fig. 5 *a* and *b*) than when grown in MCDB 170 plus 1% fetal bovine serum (peak 2 in *a* and peak 1 in *c*) or in DFCI-1 (peak 2 in *b* and *c*). However, retention of dye by 184 cells is the same in MCDB 170 plus 1% serum as in DFCI-1, which also contains 1% serum (Fig. 5*c*). Of most importance, when both normal and tumor cells are grown in DFCI-1 [compare peak 1 (normal) with peak 2 (ZR-T in *d* and 21PT in *e*), the differential retention is clearly adequate for identification of tumor cells and to use as a basis for separation by cell sorting.

DISCUSSION

We describe here a medium, DFCI-1, that supports the long-term growth in culture of human primary mammary tumor cells, of normal epithelial cells from mammoplasty, and of mammary tumor cell lines. The tumor cells are shown to be distinguishable from normal epithelial cells by morphology, by growth requirements, and by two markers: preferential expression of the HMFG-2 epitope on tumor cells and preferential retention in tumor cell mitochondria of the lipophilic dye R-123.

A single medium for growth of both normal and tumor cells has the great advantage that it can be used in comparative studies of gene expression. For example, expression of several oncogenes and growth factors was compared in normal and tumor cells, revealing that the levels of mRNA for transforming growth factor α and for epidermal growth factor receptor are lower in estrogen receptor-positive mammary tumor cell lines than in either estrogen receptor-negative tumor cells or normal cells (22).

The search for antigenic determinants on mammary epithelium to distinguish between normal and tumor cells has been an ongoing effort in many laboratories for several years (9, 11, 21, 23, 24), but a growth medium to permit comparative antibody binding studies in cell culture has not been available. The pronounced effect of DFCI-1 medium on the binding activity of DF3 and CEA antibodies, but not of HMFG-2, suggests that serum concentration may be a major variable (Fig. 4 and unpublished data). Thus DFCI-1 medium should contribute to improved predictability in testing of new antibody preparations for differential binding.

This laboratory has been engaged in studies designed to screen for tumor-suppressor genes by using subtractive hybridization (25) to isolate mRNAs expressed by normal cells but not by related tumor cells from mammary epithelium. For these experiments, it has been essential to grow both normal and tumor cell populations under the same

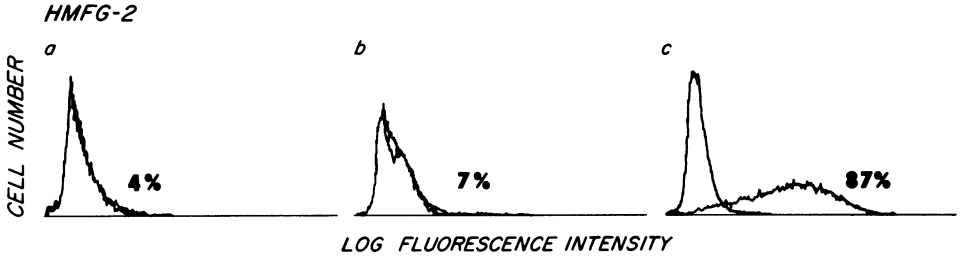

FIG. 4. Expression of HMFG-2 antigenic determinant on normal and tumor cells. Exponentially growing 76N (*a*), 21N (*b*), and 21PT (*c*) cells were treated with monoclonal antibody against HMFG-2 antigenic determinant and with control antibody as described for Fig. 3.

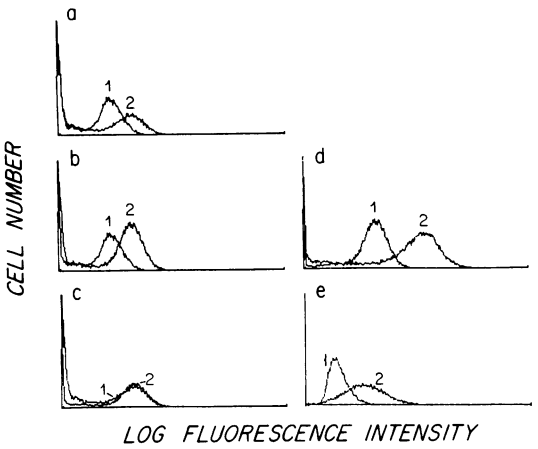

FIG. 5. Comparison of R-123 retention by normal cells and tumor cell lines. R-123 retention was measured after 48 hr in trypsinized cells by cytofluorography. (*a*) Strain 184 cells grown in serum-free MCDB (peak 1) or in MCDB plus 1% fetal bovine serum (peak 2). (*b*) Strain 184 cells grown in serum-free MCDB (peak 1) or in DFCI-1 (peak 2). (*c*) Strain 184 cells grown in MCDB plus 1% serum (peak 1) or in DFCI-1 (peak 2). (*d*) ZR-T (peak 2) with 184 (peak 1), both grown in DFCI-1. (*e*) 21PT (peak 2) with 76N (peak 1), both grown in DFCI-1.

conditions, using DFCI-1 medium. Among the genes expressed in normal mammary epithelial cells but not in breast carcinomas are keratins 5 and 6 (D. Trask, P. Yaswen, and R.S., unpublished data) and the *gro* gene, which is expressed in a minority of carcinomas (26).

In addition to its superior ability to support growth, DFCI-1 medium also offers other advantages. The salts are readily available and the pH can be maintained reliably with 6.5% CO_2. MCDB 170 requires 2% CO_2 (14) and a constant pH during growth that is very difficult to maintain. This medium has not yet been prepared satisfactorily as a dry powder. While MCDB 170 is excellent for cloning normal cells, it does not support the high-density growth required for biochemical studies.

Clearly, the most encouraging feature of the DFCI-1 medium is its ability to support the long-term growth of primary tumor cells (e.g., 21PT). These cells initially grew much more slowly than the associated normal cells (21N). However, after a few passages, the 21PT cells grew well, with a doubling time of *ca.* 2 days, and are now at passage 18-20. Some modifications of the DFCI-1 formulation may be necessary for growth of certain tumor types and to improve growth rates. Nonetheless, the fact that the 21PT tumor of origin was an infiltrating and intraductal carcinoma, a relatively frequent tumor type, supports our preliminary evidence that many other primary tumor specimens are also growing in this medium.

In summary, successful growth of primary tumor cells and tumor cell lines in DFCI-1 medium provides the basis for their characterization and for comparative studies. Fluorescence-activated cell sorting with HMFG-2 antibodies and with R-123 provides a powerful method for separation of normal and tumor cells grown from surgical specimens. With these tools in hand, many primary mammary tumors should soon be accessible to investigation.

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